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(57) Abstract

The present invention relates to a method of inhibiting cap-independent protein synthesis of eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to said eukaryotic cells. There is also disclosed a method of inhibiting translation of HIV TAR-containing messenger ribonucleic acids (mRNA) of HIV infected eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to said eukaryotic cells. There is also disclosed a composition for gene therapy using glycosaminoglycan N-acetylglycosaminyl N-deacetylase/N-sulfotransferase cDNA.

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(57) Abstract

The present invention relates to a method of inhibiting cap-independent protein synthesis of eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to said eukaryotic cells. There is also disclosed a method of inhibiting translation of HIV TAR-containing messenger ribonucleic acids (mRNA) of HIV infected eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to said eukaryotic cells. There is also disclosed a composition for gene therapy using glycosaminoglycan N-acetylglycosaminyl N-deacetylase/N-sulfotransferase cDNA.

INHIBITION OF CAP-INDEPENDENT PROTEIN SYNTHESIS BY HEPARIN OR HEPARIN MIMETICS THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the use of heparin and heparin mimetic compounds thereof to inhibit cap-independent protein synthesis (or translation) in addition to cap-dependent protein synthesis and to inhibit translation of HIV TAR-containing messenger ribonucleic acids (mRNA) mediated.

(b) Description of Prior Art

Heparin is among the best studied glycosaminoglycans. This compound is known for its involvement in a variety of physiological processes. It is involved in the control of homeostasis, smooth muscle proliferation, growth factors activity, extracellular matrix integrity, among others (Margalit, H. et al. (1993) *Journal of Biological Chemistry* **268**: 19228-19231). Heparin is a negatively charged polymer of a regular disaccharide repeat sequence with a high degree of sulfatation. Thus, many proteins are expected to bind heparin via electrostatic interactions, but electrostatic forces by themselves are probably not sufficient (Margalit, H. et al. (1993) *Journal of Biological Chemistry* **268**: 19228-19231).

Recently, the substitution of heparin for double-stranded (ds) RNA in the autophosphorylation of the interferon-inducible, RNA-dependent eIF-2α protein kinase (PKR) has been analyzed in detail (George, C.X. et al. (1996) *Virology* **221**: 180-188). Phosphorylation of the alpha subunit of eIF-2 leads to the inhibition of the cap-dependent translation (*In: Translational control* (1996). Edited by John W.B. Hershey et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA. pp. 31-69 and 139-172).

Internal initiation, or cap-independent translation, is known to occur with picornaviruses (such as poliovirus or encephalomyocarditis virus) mRNAs (*In: Translational control* (1996) Edited by John W.B. Hershey et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA. pp. 549-573), other RNAs such as BiP mRNA or Antennapedia mRNA (*In: Translational control* (1996) Edited by John W.B. Hershey et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA. pp. 95-96), and might be involved during human immunodeficiency virus (HIV) infection (Svitkin et al. (1994) *Journal of Virology* **68**, 7001-7007). Internal translation requires an internal ribosome entry site (IRES) on the mRNA. Recently, the inhibition of IRES-mediated translation of poliovirus has been shown to be inhibited by a 60-nucleotide long yeast RNA (Das., S. et al. (1996) *Journal of Virology* **70**: 1624-1632). To my knowledge, this is the only scientific report that has been published to date showing the specific and direct inhibition of cap-independent translation by a molecule, here an RNA.

Moreover, translation of mRNAs of human immunodeficiency virus type 1 (HIV-1) has been shown to be mediated by cis-acting sequences responsive to the *tat* gene product, the *trans*-acting responsive (TAR) region, which is located immediately adjacent to the site of transcription initiation (Rosen, C.A. et al. (1985) *Cell*, **41**:813-823). The TAR sequence is therefore located at the 5' end of all viral mRNAs. It has been proposed that the TAR sequence and flanking 3' region played a role in the regulation of translation of HIV-1 mRNAs by inhibiting this translation (Parkin, N.T. et al. (1988) *EMBO Journal*, **7**:2831-2837). However, in the latter study, the authors, by translating HIV-1 TAR-containing mRNAs in a rabbit reticulocyte lysate or in

a cytoplasmic extract of eukaryotic HeLa cells that have grown in suspension cultures, or by microinjecting HIV-1 TAR-containing mRNAs in Xenopus oocytes, predict that the block to translation of viral mRNAs by their 5' untranslated region (UTR) must somehow be overcome to allow for efficient viral structural protein synthesis and viral replication during viral infection (Parkin, N.T. et al. (1988) *EMBO Journal*, **7**:2831-2837).

It would be highly desirable to be provided with means to inhibit cap-independent protein synthesis or translation.

It would be highly desirable to be provided with means to inhibit translation of HIV TAR-containing messenger ribonucleic acids (mRNA).

SUMMARY OF THE INVENTION

Heparin inhibits cap-independent translation, in addition to bind to and activate PKR and therefore inhibit cap-dependent translation. This inhibition of cap-independent translation does not necessarily imply the involvement of PKR.

One aim of the present invention is to provide means to inhibit cap-independent protein synthesis or translation.

One aim of the present invention is to provide means to inhibit translation of HIV TAR-containing messenger ribonucleic acids (mRNA).

Another aim of the present invention is to provide means to inhibit cap-independent protein synthesis or translation in addition to cap-dependent protein synthesis.

In accordance with the present invention there is provided the use of heparin and heparin mimetic compounds thereof to inhibit cap-independent protein synthesis or translation in addition to cap-dependent protein synthesis.

In accordance with the present invention there is provided the use of heparin and heparin mimetic compounds thereof to inhibit translation of HIV TAR-containing messenger ribonucleic acids (mRNA).

In accordance with the present invention there is provided a method of inhibiting cap-independent protein synthesis of eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to the eukaryotic cells.

In accordance with the present invention there is provided a method of inhibiting translation of HIV TAR-containing messenger ribonucleic acids (mRNA) of HIV infected eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to the infected eukaryotic cells. Such a heparin mimetic include, without limitation, sulfated polysaccharides.

In accordance with the present invention there is provided a pharmaceutical composition for the therapeutical inhibition of cap-independent protein synthesis in a patient suffering from a viral infection, which comprises a therapeutic amount of heparin and/or heparin mimetics thereof in association with a pharmaceutical carrier. The carrier may be a liposome or a biovector.

In accordance with the present invention there is provided a pharmaceutical composition for the therapeutical inhibition of translation of HIV TAR-containing messenger ribonucleic acids (mRNA) in a patient HIV-infected, which comprises a therapeutic amount of heparin and/or heparin mimetics thereof in association with a pharmaceutical carrier. The carrier may be a liposome or a biovector.

In accordance with the present invention there is provided a composition for gene therapy of patients infected with a virus, which comprises administering to

the patient an expression vector consisting of a cDNA sequence coding for enzyme glycosaminoglycan N-acetyl-glucosaminyl N-deacetylase/N-sulfotransferase with a constitutive or inducible promoter operatively linked upstream of the cDNA sequence, the expression vector is adapted to express the enzyme thereby reducing viral protein synthesis in the patient.

The expression vector may be a recombinant virus selected from the group consisting of adenovirus and retrovirus for targeting of the infected cells.

The delivery vector may be a liposome for targeting of the infected cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a fluorography of radiolabelled polypeptides synthesized *in vitro* in a rabbit reticulocyte lysate in accordance with the method of the present invention;

Fig. 2 illustrates a fluorography of radiolabelled polypeptides synthesized *in vitro* in a Krebs ascites fluid in accordance with the method of the present invention;

Fig. 3 illustrates a fluorography of radiolabelled polypeptides synthesized *in vitro* in a cytoplasmic extract obtained from eukaryotic cells in accordance with the method of the present invention;

Fig. 4 illustrates a fluorography of radiolabelled polypeptides synthesized *in vitro* in a cytoplasmic extract obtained from eukaryotic cells that have grown as monolayers; and

Fig. 5 illustrates a scheme of potential targeting of virally infected cells using heparin or heparin mimetics thereof in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, the use of heparin inhibits:

(i) cap-independent translation and,

(ii) translation of mRNAs containing the TAR structure from HIV-1 at their 5' end,

in addition to bind to and activate the double-stranded (ds) RNA-dependent protein kinase PKR. This inhibition of cap-independent translation and HIV TAR-mediated translation does not necessarily imply the involvement of PKR.

In accordance with the present invention there is provided a method of inhibiting cap-independent protein synthesis of eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to the eukaryotic cells.

In accordance with the present invention there is provided a method of inhibiting translation of mRNAs containing the TAR structure from HIV-1 at their 5' end, which comprises adding heparin or heparin mimetics thereof to the eukaryotic cells.

In accordance with the present invention, the method of inhibiting translation of mRNAs containing the TAR structure is intended to be used also for HIV-2, since HIV-2 has also a TAR structure.

In accordance with the present invention there is provided a pharmaceutical composition for the therapeutical inhibition of cap-independent protein synthesis in a patient suffering from a viral infection, which comprises a therapeutic amount of heparin and/or heparin mimetics thereof in association with a pharmaceutical carrier. The carrier may be a liposome or a biovector.

In accordance with the present invention there is provided a pharmaceutical composition for the thera-

peutical inhibition of TAR (HIV-1) synthesis in a HIV-1 infected patient, which comprises a therapeutic amount of heparin and/or heparin mimetics thereof in association with a pharmaceutical carrier. The carrier may be a liposome or a biovector.

Materiel and methods

A) Inhibition of cap-independent translation

In vitro protein syntheses were performed by employing a rabbit reticulocyte lysate from commercial source (Promega), and a Krebs ascites fluid, by following the manufacturer's instructions or currently employed procedures, respectively. Furthermore, a cytoplasmic extract obtained from eukaryotic cells has been generated by following a method described by Skup, D. et al. ((1977) *Nucleic Acids Research* **4**: 3581-3587). To label the newly-synthesized polypeptides during 1-hour-incubation reactions, ^{35}S-methionine (Amersham; >1200 Ci/mmol) was employed. Heparin, which was resuspended in phosphate-buffered saline (PBS) and stored at room temperature, was obtained from a commercial source (Gibco BRL; catalog number 15077-019; 100000 units at 164 units per mg). Messenger RNAs were either a) transcribed *in vitro* for the generation of a capped CAT (chloramphenycol)-EMC (IRES of encephalomyocarditis virus)-LUC (luciferase) mRNA as previously described (Pause, A. et al. (1994) *Nature* **371**: 762-767), or b) from viral source, i.e.: from poliovirus (Mahoney strain) and from encephalomyocarditis virus. Analysis of the labeled polypeptides was performed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels were treated for fluorography with En$_3$Hance (Dupont).

B) Inhibition of translation of mRNAs containing the TAR structure from HIV-1 at their 5' end

The cytoplasmic extract employed for translation was obtained from monkey Cos-1 cells that have grown as monolayers.

To label the newly-synthesized polypeptides during 1-hour-incubation reactions, ^{35}S-methionine (Amersham; >1200 Ci/mmol) was employed. Heparin, which was resuspended in phosphate-buffered saline (PBS) and stored at room temperature, was obtained from a commercial source (Gibco BRL; catalog number 15077-019).

HIV-1 TAR-containing mRNA is capped TAR(+111)CAT RNA described by Parkin, N.T. et al. ((1988) *EMBO Journal*, **7**:2831-2837). The 5' region of the latter mRNA corresponds to nucleotides +1 to +111 of HIV mRNAs and is transcribed from plasmid pSP64/TAR(+111)CAT.

Analysis of the labeled polypeptides was performed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels were treated for fluorography with En$_3$Hance (Dupont).

Results

A) Translation in rabbit reticulocyte lysate and in Krebs ascites fluid.

As shown in the fluorography of Fig. 1 (rabbit reticulocyte lysate translation) and Fig. 2 (translation in Krebs ascites fluid), inhibition of both cap-dependent translation (as seen with incorporation of ^{35}S-methionine into the CAT polypeptide) and cap-independent translation (as seen with incorporation of ^{35}S-methionine into the LUC polypeptide) is reduced, an event dependent of the heparin concentration present in the reaction. Furthermore, translation of both the poliovirus and encephalomyocarditis virus polypeptide precursors are drastically reduced when 5 µg per µl of heparin is present in the translation reaction, when compared to the reactions that contain the viral RNAs

alone. Both extracts have been previously treated with micrococcal nuclease to hydrolyze the endogenous mRNAs, and the micrococcal nuclease has been inhibited with EGTA or pTp (2'deoxythymidine, 3'-5'-diphosphate) prior to the use of the extracts. 0.4 µg of CAT-EMC-LUC RNA and 0.2 µg of either EMC or poliovirus RNA were employed in 40 µl translation reactions.

B) Inhibition of cap-dependent translation in a cell extract obtained from eukaryotic cells that have grown in suspension cultures

A cytoplasmic extract from BHK (baby hamster kidney) cells has been generated and in vitro translation reactions have been performed by following the method described by Skup and Millward (Skup, D. et al. (1977) *Nucleic Acids Research* **4**: 3581-3587). The cytoplasmic extract has not been treated with micrococcal nuclease. Again, a drastic inhibition of both cap-dependent and cap-independent translation is observed, as mentioned above in section A): as seen on this autoradiogram (Fig. 3), 5 µg per µl of heparin totally abolishes translation of the CAT (translated in a cap-dependent fashion) and the LUC polypeptides (translated in a cap-independent fashion) in the translation reaction. This reveals that the initiation of their translation is inhibited. Translation of the latter polypeptides is recovered accordingly when the concentration of heparin is reduced in the reactions. 0.4 µg of CAT-EMC-LUC RNA was employed in 40 µl translation reactions.

C) Inhibition of HIV-1 TAR-mediated translation in a cytoplasmic extract obtained from cells that have grown as monolayers

A cytoplasmic extract from monkey Cos-1 cells has been generated. The cytoplasmic extract has not been treated with micrococcal nuclease.

Interestingly, the capped TAR(+111)CAT RNA is very efficiently translated in this system (Fig. 4; lane 2), when compared to the control reaction that was performed without exogenously added RNA (lane 1). This observed efficient synthesis of capped TAR(+111)CAT RNA is thus in contrast to the results obtained by others (Parkin, N.T. et al. (1988) *EMBO Journal*, **7**:2831-2837). However, the translation of the capped TAR(+111)CAT RNA is drastically inhibited by heparin (lane 3: 1.25 µg per µl of translation reaction; lane 4: 0.125 µg per µl; lane 5: 0.0125 µg per µl; lane 6: 0.00125 µg per µl; in the latter case, translation of capped TAR(+111)CAT RNA is inhibited by more than 90% when compared to the translation performed in absence of heparin as shown in lane 2). Translation of capped TAR(+111)CAT RNA recovers when heparin is present at a concentration of 0.000125 µg per µl (lane 7).

0.4 µg of capped TAR(+111)CAT RNA was employed in 40 µl translation reactions (lanes 2 to 7).

Discussion

To date, virus replication inhibition by heparin or heparin mimetic compounds thereof have been shown to be restricted to the virus adsorption stage of the virus to the target cells (Taylor, D.L. et al. (1995) *Antiviral Research* **28**: 159-173; Thormar, H. et al. (1995) *Antiviral Research* **27**: 49-57; Banfield, B.W. et al. (1995) *Virology* **208**: 531-539; Ida, H. et al. (1994) *Antiviral Research* **23**: 143-159; Barzu, T. et al. (1993) *Journal of medicinal Chemistry* **36**: 3546-3555; Hanssens, F.P. et al. (1993) *Journal of Virology* **67**: 4492-4496). One report mentions the *in vitro* inhibition of translation of brome mosaic virus RNA in a wheat germ extract in presence of heparin, however an effect mediated by the plant homologue of PKR (pPKR) (Langland, J.O. et al. (1996) *Plant Physiology and Biochemistry* **34**: 521-

526). To my knowledge, no report has been thus far published showing inhibition of cap-independent translation and HIV TAR-mediated translation by heparin or heparin mimetics either *in vitro* or *in vivo*.

Thus, my discovery has a great potential when one considers the fact that heparin or heparin mimetics can be targeted by different means to cells that have been infected by viruses, the latter showing a cap-independent translation initiation and HIV TAR-mediated of their RNA. This targeting of heparin might be performed and mediated by biovectors or liposomes, for example, the latter vectors containing heparin or heparin mimetics. This targeting can be specific when one considers the strategy depicted in Fig. 5.

Perspectives

1) Potentially, any kind of cap-independent translation resulting on the attachment on RNA of ribosomes by internal ribosome entry could be reduced or inhibited with adequate concentrations of heparin or heparin mimetics thereof.

2) The effect of small heparin oligosaccharides of define lengths (as described in George, C.X. et al. (1996) *Virology* **221**: 180-188) should be analyzed for their ability to reduce or inhibit the extent of cap-independent translation.

3) The effect of heparin or heparin mimetics on HIV-2 TAR-mediated translation of RNA should be analyzed.

4) Some experiments should be performed *in vivo* as well as *in situ*: heparin should be incorporated into liposomes or biovectors which are then targeted to cells infected with various viruses by following the general procedure depicted in Fig. 5.

5) Targeting of any kind cell, not necessarily virus-infected, in which the cap-independent translation of any protein has to be inhibited.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Therapeutic treatment of virus-infected cells

As shown in Fig. 5A, target cells are infected by viruses through ligand-receptor interactions. As depicted, receptors are on the surface of the virus-infected cells and are recognized by the ligand of the virus.

To generate newly synthesized virus particles, the ligand(s) of the virus has(ve) to be exposed at the cell surface of the infected cells (Fig. 5B).

To selectively target the virus-infected cells liposomes or biovectors containing heparin and/or heparin mimetics and bearing the cell receptor at their surface will be generated. These liposomes or biovectors will selectively interact with the virus-infected cells through ligand-receptor interactions and will allow the transfer of the heparin and/or heparin mimetics into the virus-infected cells. This will inhibit the viral protein synthesis and render the virus to be avirulent (unable to synthesize the viral components, such as viral proteins).

The above-described therapeutic treatment of virus-infected cells will be effective for any virus wherein its protein synthesis is cap-independent.

EXAMPLE II

Gene therapy using glycosaminoglycan N-acetylglucosaminyl N-deacetylase/N-sulfotransferase cDNA

Heparin biosynthesis occurs only in connective tissue mast cells (Nader, H.B., and Dietrisch, in Heparin; Lane, D.A. and Lindahl, U, eds pp. 81-96, Arnold, London). The biosynthesis of heparin is initiated by glycosylation reactions that generate saccharide sequences composed of alternating D-glucuronic and N-acetylglucosamine (GlcNAc) units. N-deacetylation/N-sulfation of N-acetylglucosamine is a key event in the biosynthesis of heparin. This N-deacetylation/N sulfation of GlcNAc is an obligatory step for the subsequent reactions for the biosynthesis of heparin.

Recently, cDNAs coding for enzyme containing N-deacetylase/N-sulfotransferase activities have been cloned from a heparin-producing cell line MST from mouse (Orellana, A., Hirschberg, C.B., Wei, Z., Swiedler, S.J., and Ishihara M., Journal of Biological Chemistry 21, pp. 2270-2276, 1994) and from a mouse mastocytoma cell line (Erikson, I., Sandbäck, D., Ek, B., Lindhal, U., and Kjellen, L., Journal of Biological Chemistry 14, pp. 10438-10443, 1994).

In order to produce heparin in cell lines that do not normally produce heparin, it might be of great interest to express an enzyme that contains glycosaminoglycan N-acetylglucosaminyl N deacetylase/N-sulfotransferase activities in these cells. For this, the cDNA coding for a glycosaminoglycan N-acetylglucosaminyl N-deacetylase/N-sulfotransferase is incorporated into a plasmid or any suitable DNA sequence under the control of a viral or an eukaryotic promoter, thus allowing transcription of the corresponding RNA in these cells. This promoter can be an inducible promoter or a constitutive promoter. Targeting of the cells in order to incorporate the glycosaminoglycan N-acetylglu-

cosaminyl N-deacetylase/N-sulfotransferase cDNA can be performed by gene therapy using retroviral vectors, adenoviral vectors, or liposome-mediated gene delivery.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

I CLAIM:

1. A method of inhibiting cap-independent protein synthesis of eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to said eukaryotic cells.

2. The method of claim 1, wherein said heparin mimetic is sulfated polysaccharides.

3. A method of inhibiting translation of HIV TAR-containing messenger ribonucleic acids (mRNA) of HIV infected eukaryotic cells, which comprises adding heparin or heparin mimetics thereof to said eukaryotic cells.

4. A pharmaceutical composition for the therapeutical inhibition of cap-independent protein synthesis in a patient suffering from a viral infection, which comprises a therapeutic amount of heparin and/or heparin mimetics thereof in association with a pharmaceutical carrier.

5. The composition of claim 4, wherein said carrier is a liposome or a biovector.

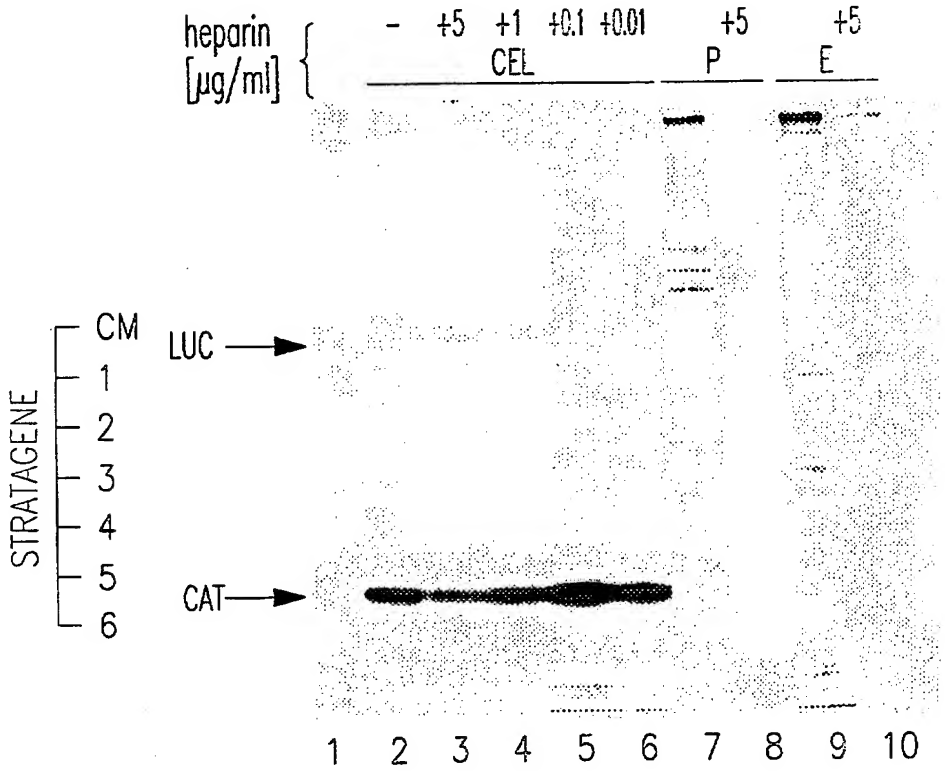
6. A pharmaceutical composition for the therapeutical inhibition of translation of HIV TAR-containing messenger ribonucleic acids (mRNA) in a HIV-infected patient, which comprises a therapeutic amount of heparin and/or heparin mimetics thereof in association with a pharmaceutical carrier.

7. The composition of claim 6, wherein said carrier is a liposome or a biovector.

8. A composition for gene therapy of patients infected with a virus, which comprises administering to said patient an expression vector consisting of a cDNA sequence coding for enzyme glycosaminoglycan N-acetyl-glucosaminyl N-deacetylase/N-sulfotransferase with a constitutive or inducible promoter operatively linked upstream of the cDNA sequence, said expression vector is adapted to express said enzyme thereby reducing viral protein synthesis in said patient.

9. The composition of claim 8, wherein the expression vector is a recombinant virus selected from the group consisting of adenovirus and retrovirus for targeting of the infected cells.

10. The composition of claim 8, wherein the delivery vector is a liposome for targeting of the infected cells.



P: +poliovirus RNA

E: +encephalo-myocarditis virus RNA

Reticulocute lysate
12.5% SDS-PAGE
Exp. 16.8

CEL: +CAT-EMC-LUC RNA

18 h exposure

Fig. 1

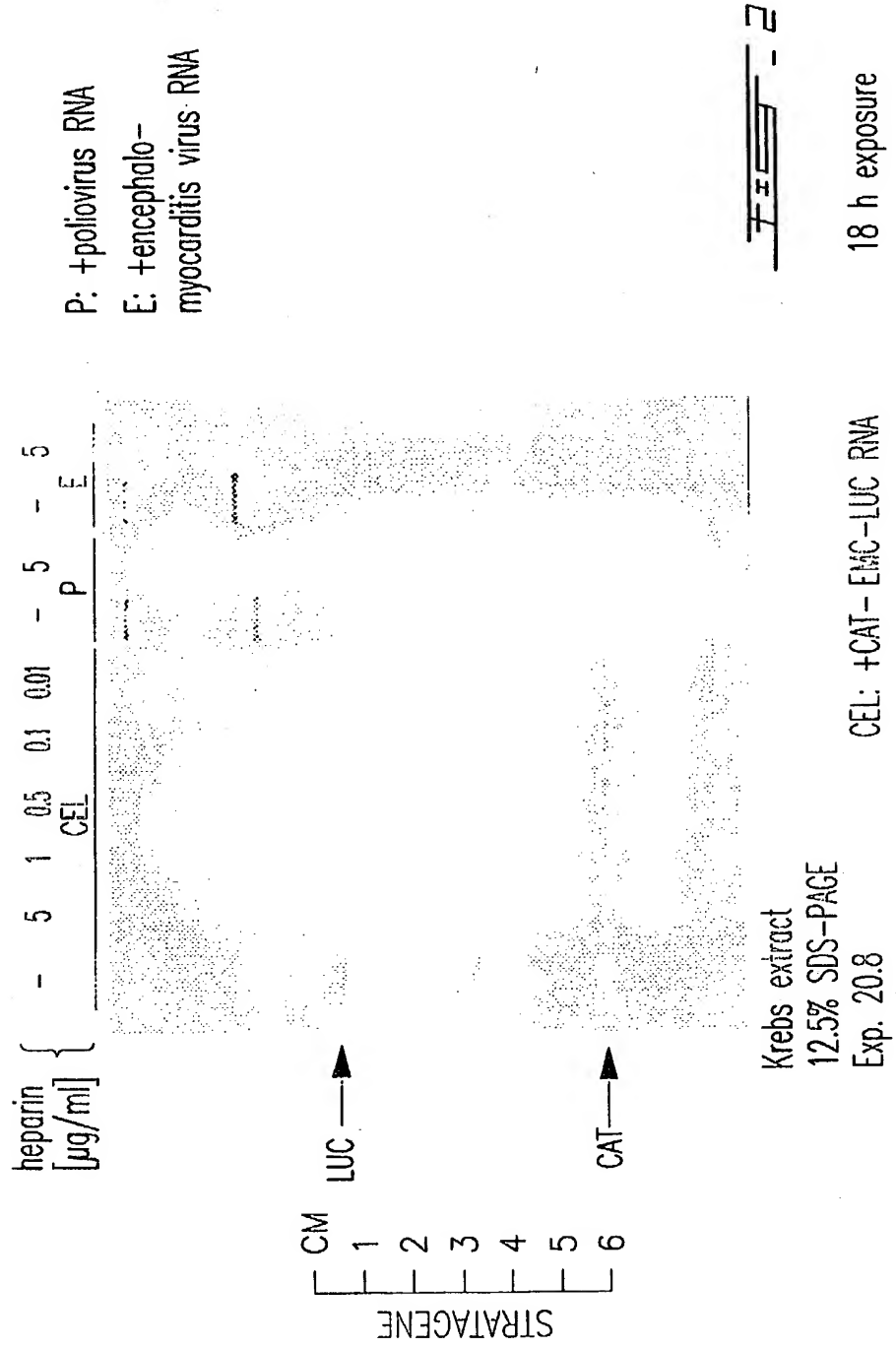

heparin [µg/ml]
- 5 1 0.5 0.1 0.01 - 5 - 5
CEL
P
E
P: +poliovirus RNA
E: +encephalo-myocarditis virus RNA
LUC
CAT
CM 1 2 3 4 5 6
STRATAGENE
Krebs extract
12.5% SDS-PAGE
Exp. 20.8
CEL: +CAT- EMC-LUC RNA
18 h exposure

Fig - 2

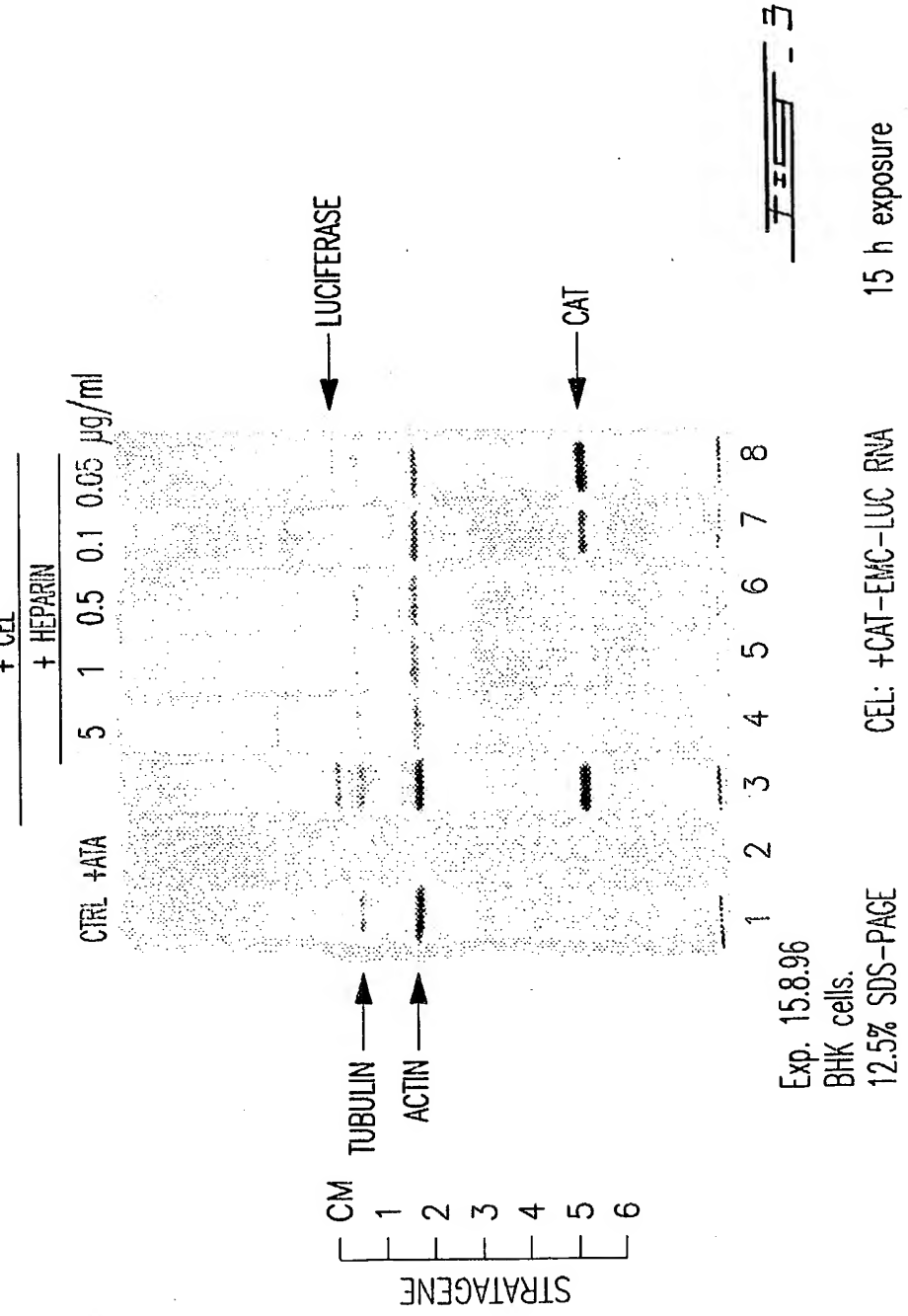

+ CEL
+ HEPARIN
CTRL +ATA 5 1 0.5 0.1 0.05 µg/ml
LUCIFERASE
CAT
TUBULIN
ACTIN
1 2 3 4 5 6 7 8
CM 1 2 3 4 5 6
STRATAGENE
Exp. 15.8.96
BHK cells.
12.5% SDS-PAGE
CEL: +CAT-EMC-LUC RNA
15 h exposure

Fig - 3

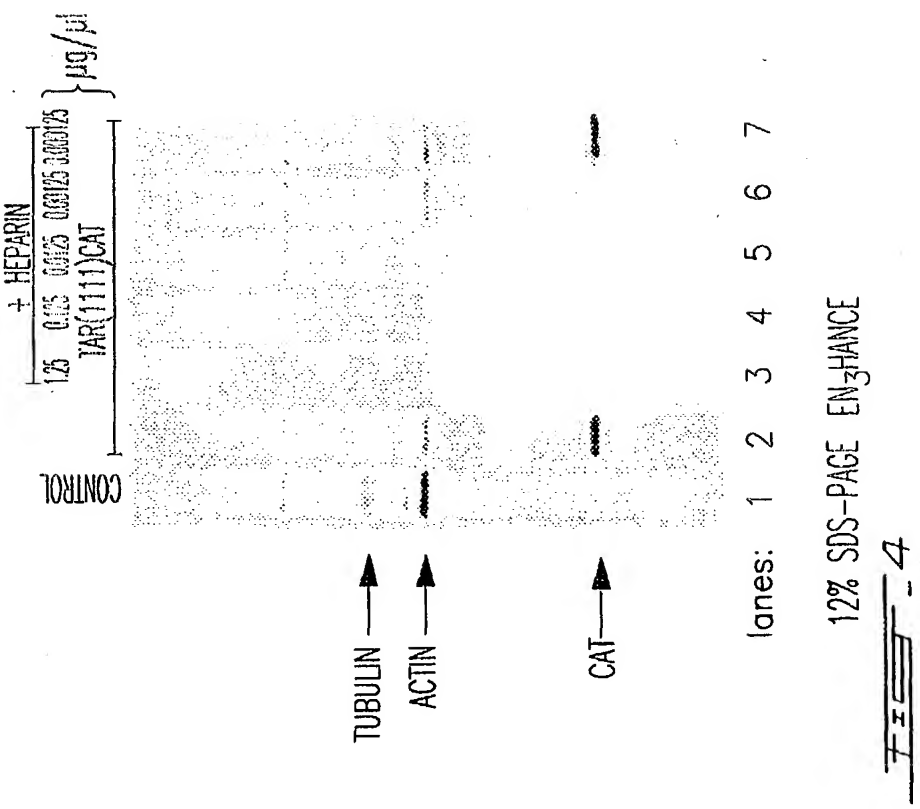
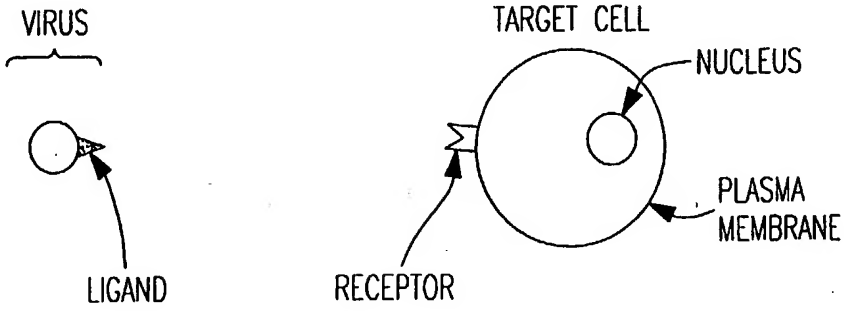
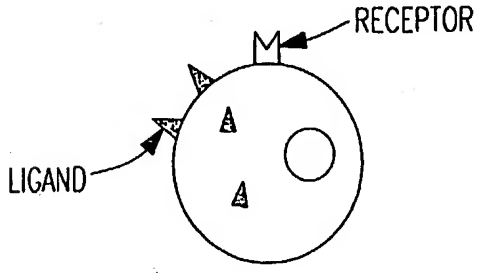

CONTROL
+ HEPARIN
TAR(1111)CAT
125 0.125 0.0625 0.03125 0.015625
μg/μl
TUBULIN
ACTIN
CAT
lanes: 1 2 3 4 5 6 7
12% SDS-PAGE EN3HANCE
Cos1 cell extract (cytoplasmic)
"thawed"
48 h exposure

Fig. 4

A: RECOGNITION OF A TARGET CELL BY LIGAND-RECEPTOR INTERACTION



B: VIRALLY-INFECTED CELLS PRODUCE LIGAND THAT IS PRESENT AT THE CELL-SURFACE:



C: TARGETING OF VIRALLY-INFECTED CELLS WITH (FOR EXAMPLE) LIPOSOMES OR BIOVECTORS BEARING THE RECEPTOR ON THEIR SURFACE:

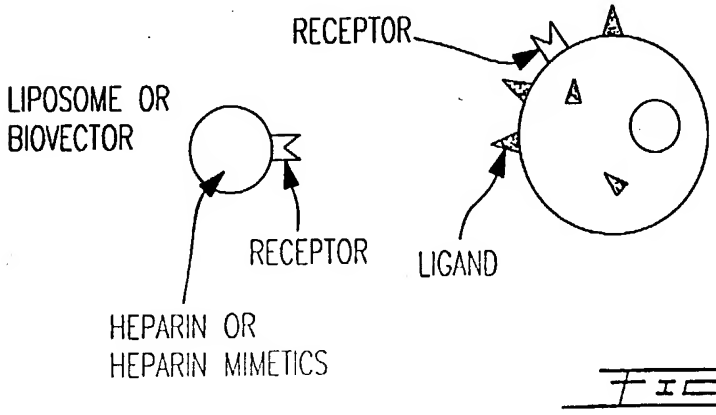


FIG. 5

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/CA 97/00794

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/715 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANALAYSAY ET AL.: "Inhibition of Mesothelial Cell Growth and Protein Synthesis by Heparin" ADV. PERITON. DIAL., vol. 11, 1995, pages 239-242, XP002056007 see abstract; figure 2 ---	1
X	WACKER ET AL.: "Spezifische und unspezifische Hemmung der zellfreien Proteinsynthese mit Polyanionen" Z. NATURFORSCH. B, vol. 22, no. 4, 1967, pages 413-417, XP002056008 see tables 1-4 ---	1,2
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 1998	23.06.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer A. Jakobs

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INTERNATIONAL SEARCH REPORT

Interna[illegible]nal Application No
PCT/CA 97/00794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANNAVAJJALA ET AL.: "CELL SURFACE HEPARIN PROTEOGLYCAN MEDIATE ATTACHMENT TO BASIC DOMAIN OF HIV-1 TAT PROTEIN" AIDS RES. HUMAN RETROV., vol. 10, no. suppl. 3, 1994, page s77 XP002056009 see the whole document ---	3,4,6
X	WALDMAN ET AL.: "Heparin as inhibitor of mammalian protein synthesis. II. Degree of sulfation. Related sulfated mucopolysaccharides" BIOCHIM. BIOPHYS. ACTA, vol. 343, no. 2, 1974, pages 324-329, XP002056010 see abstract; figure 1; tables 1,2 ---	1,2
X	HALPER ET AL.: "Modulation of Growth of Human Carcinoma SW-13 Cells by Heparin and Growth Factors" J. CELL. PHYS., vol. 141, no. 1, 1989, pages 16-23, XP002056011 see tables 1,3 ---	1,2
X	REILLY ET AL.: "Rat Vascular Smooth Muscle Cells Immortalized with SV40 Large T antigen Possess Defined Smooth Muscle Inhibition by Heparin" J. CELL. PHYS., vol. 142, no. 2, 1990, pages 342-351, XP002056012 see page 346, column 2, paragraph 3 - page 347, column 1, paragraph 1; figure 6 ---	1
X,P	WITVROUW ET AL.: "Sulfated polysaccharides Extracted from Sea Algae as Potential Antiviral Drugs" GEN. PHARMAC., vol. 29, no. 4, 1997, pages 497-511, XP002056013 see abstract ---	3,4,6
X	MEYLAN ET AL.: "Influence of Host Cell Type and V3 Loop of the Surface Glycoprotein on Susceptibility of Human Immunodeficiency Virus Type 1 to Polyanion Compounds" ANTIMICROBIAL AGENTS CHEMOTHER., vol. 38, no. 12, 1994, pages 2910-2916, XP002056014 see page 2910, column 1, paragraph 1; figures 1,3,4 --- -/--	3,4,6

2

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/CA 97/00794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOWELL ET AL.: "LOW MOLECULAR WEIGHT HEPARIN INHIBITS INFECTIVITY AND PREVENTS REPLICATION OF HIV-1 IN T CELLS" BLOOD, vol. 84, no. 10 suppl. 1, 1994, page 701a XP002056015 see the whole document	1-4,6
X	GREENBERG ET AL.: "Effect of Protein Synthesis Inhibitors on Growth Factor Activation of c-fos, c-myc, and Actin Gene Transcription" MOL. CELL. BIOL., vol. 6, no. 4, 1986, pages 1050-1057, XP002056016 see page 1054, column 1, line 11 - line 15; figure 6	1
X	DATABASE WPI Section Ch, Week 9614 Derwent Publications Ltd., London, GB; Class A96, AN 96-136210 XP002056017 & JP 08 027 030 A (TERUMO CORP) , 30 January 1996 see abstract	4-7
X	DATABASE WPI Section Ch, Week 9531 Derwent Publications Ltd., London, GB; Class B04, AN 95-237131 XP002056018 & JP 07 145 038 A (TERUMO CORP) , 6 June 1995 see abstract	4-7
X	DATABASE WPI Section Ch, Week 8505 Derwent Publications Ltd., London, GB; Class B05, AN 85-027827 XP002056019 & JP 59 222 410 A (TERUMO CORP) , 14 December 1984 see abstract	4-7
X	WO 92 13524 A (NATTERMANN A & CIE) 20 August 1992 see example 1	4-7
X	DE 29 07 303 A (PAPAHADJOPOULOS DEMETRIOS P) 6 September 1979 see page 21, paragraph 3	4-7

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 97/00794

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Subject 1: Claims 1-7
Subject 2: Claims 8-10

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna...al Application No
PCT/CA 97/00794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9213524 A	20-08-92	DE 4121389 A	13-08-92
		EP 0527979 A	24-02-93
DE 2907303 A	06-09-79	US 4235871 A	25-11-80
		BE 874408 A	23-08-79
		EP 0004223 A	19-09-79
		FR 2418023 A	21-09-79
		GB 2015464 A,B	12-09-79
		US 4394448 A	19-07-83
		US 4394149 A	19-07-83

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Interna # Application No
PCT/CA 97/00794

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/715 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANALAYSAY ET AL.: "Inhibition of Mesothelial Cell Growth and Protein Synthesis by Heparin" ADV. PERITON. DIAL., vol. 11, 1995, pages 239-242, XP002056007 see abstract; figure 2 ---	1
X	WACKER ET AL.: "Spezifische und unspezifische Hemmung der zellfreien Proteinsynthese mit Polyanionen" Z. NATURFORSCH. B, vol. 22, no. 4, 1967, pages 413-417, XP002056008 see tables 1-4 --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 1998	23.06.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer A. Jakobs

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/CA 97/00794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANNAVAJJALA ET AL.: "CELL SURFACE HEPARIN PROTEOGLYCAN MEDIATE ATTACHMENT TO BASIC DOMAIN OF HIV-1 TAT PROTEIN" AIDS RES. HUMAN RETROV., vol. 10, no. suppl. 3, 1994, page s77 XP002056009 see the whole document ---	3,4,6
X	WALDMAN ET AL.: "Heparin as inhibitor of mammalian protein synthesis. II. Degree of sulfation. Related sulfated mucopolysaccharides" BIOCHIM. BIOPHYS. ACTA, vol. 343, no. 2, 1974, pages 324-329, XP002056010 see abstract; figure 1; tables 1,2 ---	1,2
X	HALPER ET AL.: "Modulation of Growth of Human Carcinoma SW-13 Cells by Heparin and Growth Factors" J. CELL. PHYS., vol. 141, no. 1, 1989, pages 16-23, XP002056011 see tables 1,3 ---	1,2
X	REILLY ET AL.: "Rat Vascular Smooth Muscle Cells Immortalized with SV40 Large T antigen Possess Defined Smooth Muscle Inhibition by Heparin" J. CELL. PHYS., vol. 142, no. 2, 1990, pages 342-351, XP002056012 see page 346, column 2, paragraph 3 - page 347, column 1, paragraph 1; figure 6 ---	1
X,P	WITVROUW ET AL.: "Sulfated polysaccharides Extracted from Sea Algae as Potential Antiviral Drugs" GEN. PHARMAC., vol. 29, no. 4, 1997, pages 497-511, XP002056013 see abstract ---	3,4,6
X	MEYLAN ET AL.: "Influence of Host Cell Type and V3 Loop of the Surface Glycoprotein on Susceptibility of Human Immunodeficiency Virus Type 1 to Polyanion Compounds" ANTIMICROBIAL AGENTS CHEMOTHER., vol. 38, no. 12, 1994, pages 2910-2916, XP002056014 see page 2910, column 1, paragraph 1; figures 1,3,4 --- -/--	3,4,6

2

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/CA 97/00794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOWELL ET AL.: "LOW MOLECULAR WEIGHT HEPARIN INHIBITS INFECTIVITY AND PREVENTS REPLICATION OF HIV-1 IN T CELLS" BLOOD, vol. 84, no. 10 suppl. 1, 1994, page 701a XP002056015 see the whole document	1-4,6
X	GREENBERG ET AL.: "Effect of Protein Synthesis Inhibitors on Growth Factor Activation of c-fos, c-myc, and Actin Gene Transcription" MOL. CELL. BIOL., vol. 6, no. 4, 1986, pages 1050-1057, XP002056016 see page 1054, column 1, line 11 - line 15; figure 6	1
X	DATABASE WPI Section Ch, Week 9614 Derwent Publications Ltd., London, GB; Class A96, AN 96-136210 XP002056017 & JP 08 027 030 A (TERUMO CORP) , 30 January 1996 see abstract	4-7
X	DATABASE WPI Section Ch, Week 9531 Derwent Publications Ltd., London, GB; Class B04, AN 95-237131 XP002056018 & JP 07 145 038 A (TERUMO CORP) , 6 June 1995 see abstract	4-7
X	DATABASE WPI Section Ch, Week 8505 Derwent Publications Ltd., London, GB; Class B05, AN 85-027827 XP002056019 & JP 59 222 410 A (TERUMO CORP) , 14 December 1984 see abstract	4-7
X	WO 92 13524 A (NATTERMANN A & CIE) 20 August 1992 see example 1	4-7
X	DE 29 07 303 A (PAPAHADJOPOULOS DEMETRIOS P) 6 September 1979 see page 21, paragraph 3	4-7

2

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 97/00794

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Subject 1: Claims 1-7
Subject 2: Claims 8-10

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-7

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna..ial Application No
PCT/CA 97/00794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9213524 A	20-08-92	DE 4121389 A	13-08-92
		EP 0527979 A	24-02-93
DE 2907303 A	06-09-79	US 4235871 A	25-11-80
		BE 874408 A	23-08-79
		EP 0004223 A	19-09-79
		FR 2418023 A	21-09-79
		GB 2015464 A,B	12-09-79
		US 4394448 A	19-07-83
		US 4394149 A	19-07-83